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Journal:	*Avian Pathology*
Manuscript ID:	CAVP-2008-0137
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	10-Sep-2008
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Keywords:	avian nephritis virus, chicken astrovirus, duck hepatitis virus, enterovirus-like virus

Cavp-2008-0137
(Formerly cavp-2008-0068)

Identification of chicken enterovirus-like viruses, duck hepatitis virus (DHV) type 2 and DHV type 3 as astroviruses

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Short title: Chicken and duck astroviruses

Note from EiC.

Figure 1 (immunofluorescence). Black and white is sufficient in hard copy.

Figures 3, 4a and 4b should be given as much space as they require:

Figure 3: use full width of portrait page.

Figure.4a Likewise – it will take up much of a portrait page.

Figure. 4b Likewise – will need virtually all of a portrait page.

Would be ideal if Figures 4a and 4b were on facing pages.

On page 10 and at the end of the legend to Figure 4 there is a sentence referring to supplementary data. This is a single figure that will be available online only. This supplementary figure could be referred to as Figure S1.

Received 10.9.2008

Abstract

Earlier work identified and biologically characterised antigenically distinct enterovirus-like viruses (ELVs) of chickens. Three of these ELVs can now be identified as astroviruses. Characterisation involved the use of a hitherto undescribed, degenerate primer based reverse transcription polymerase chain reaction (RT-PCR) to amplify astrovirus ORF 1b-specific cDNA fragments followed by nucleotide sequence determination and analysis of the amplified fragments. ELV-1 was confirmed as an isolate of the astrovirus avian nephritis virus (ANV). ELV-4 (isolate 612) and ELV-3 (isolates FP3 and 11672) were antigenically and genetically related to the second characterised astrovirus of chickens, namely chicken astrovirus (CAstV). Using indirect immunofluorescence, the FP3 and 11672 ELV-3 isolates were very closely related to one another and less closely related to ELV-4 and the previously described CAstV (P22 18.8.00 reference isolate). Comparative analyses based on the ORF 1b amplicon sequences showed that the FP3 and 11672 ELV-3 isolates shared high nucleotide (95%) and amino acid (98%) identities with one another, and lower nucleotide (76-79%) and amino acid (84-85%) identity levels with ELV-4 and the reference CAstV P22 18.8.00 isolates. The combined degenerate primer RT-PCR and sequencing methods also provided nucleotide sequence specific to duck hepatitis virus type 2 (DHV-2) (renamed duck astrovirus) and duck hepatitis virus type 3 (DHV-3), which, for the first time, can also be identified as an astrovirus. Phylogenetic analyses based on the amplified ORF 1b sequences showed that ANV was the most distantly related avian astrovirus, with DHV-3 being more closely related to turkey astrovirus type 2 than DHV-2.

Introduction

The detections in avian samples of small spherical viruses, measuring 25-30 nm in diameter, have been reported on numerous occasions (McNulty & Guy, 2003). They have often been

referred to as enterovirus-like viruses (ELV) on the basis of characteristics shared with viruses of the genus *Enterovirus* (family *Picornaviridae*) including size, morphology, resistance to pH3, cytoplasmic replication and detection in faeces. With several exceptions, ELVs remain largely uncharacterised and unclassified. Exceptions include avian encephalomyelitis virus (AEV), a tentative member of the genus *Hepatovirus* (family *Picornaviridae*) (Marvil et al., 1999), duck hepatitis virus type 1 (DHV-1), a novel picornavirus (Tseng *et al*., 2007), another novel duck picornavirus (Tseng & Tsai, 2007), and avian nephritis virus (ANV), characterised as a member of the genus *Avastrovirus* of the family *Astroviridae* (Imada *et al*. 2000). More recently, the characterisation of a different astrovirus of chickens, named chicken astrovirus (CAstV), was reported by Baxendale & Mebatsion (2004).

Earlier work in our laboratory resulted in the biological characterisation of four ELVs (McNulty *et al*., 1990; McNeilly *et al*., 1994), which were antigenically different from one another and from AEV, DHV-1 and duck hepatitis virus type 3 (DHV-3). ELV-1 was obtained from chickens affected by runting syndrome (McNulty *et al*., 1984); ELV-2 (isolate EF84/700) was isolated from 27-day-old normal broilers (McNulty *et al*., 1987); ELV-3 (isolate FP3) was isolated from dead-in-shell chicks during an investigation of early broiler mortality in the UK (Spackman *et al*., 1984) and ELV-4 (isolate 612) was isolated from South African broiler chickens with respiratory problems (McNeilly *et al*., 1994). On the basis of antigenic similarity shared with the G4260 serotype 1 reference strain, ELV-1 can be regarded as an ANV (McNulty *et al*., 1990). However, ELV-2, -3 and -4 remain uncharacterised.

Our interest in these viruses relates to their possible involvement in causing enteritis and growth depression in chickens (McNulty & Guy, 2003). ELV-1, -3 and -4, were shown to cause varying degrees of growth retardation following experimental infections of 1-day-old SPF or broiler chicks (McNulty *et al*., 1990; McNeilly *et al*., 1994; Smyth *et al*., 2007). However, their roles in causing disease in broiler chickens in the field are undetermined due to the absence of sensitive, specific and convenient diagnostic tests. Experience to date indicates that most ELVs do not grow productively in cell culture, and although propagation in chicken embryos is often possible this procedure is labour-intensive and time-consuming for routine diagnosis. In addition, virus-specific antisera are not commonly available for use

in immunostaining-based diagnostic procedures. The development of molecular diagnostic tests such as PCR and RT-PCR, which are becoming increasingly used in diagnostic investigations and routine diagnosis, is not possible for ELVs because genome sequence data are unavailable.

This paper describes the use of a degenerate primer-based RT-PCR method to isolate astrovirus-specific cDNAs from ELV samples. Nucleotide sequence determinations and analyses of amplified fragments have identified ELV-1, -3 and -4 as astroviruses. Nucleic acid sequence data is also presented for DHV-2 and DHV-3, which are also identified as astroviruses. The phylogenetic relationship (based on ORF 1b sequences) of the newly-characterised avian astroviruses to previously sequenced examples including ANV, and turkey astrovirus types 1 (TAstV-1) and 2 (TAstV-2) is described.

Methods and Materials

Viruses. Information relating to the source and propagation history of ELV-1, -2 and -3, which were characterised biologically in earlier work, was described by McNulty *et al*. (1990). Briefly, ELV-1 (isolate EF91-276) was available as a clarified faeces suspension, prepared from Northern Ireland broilers, 1 to 6 days of age, which subsequently developed runting-stunting syndrome (McNulty *et al.*, 1984). ELV-2 (isolate EF84/700), which was isolated in this laboratory from the faeces of a normal 27-day-old broiler, was prepared after growth in the chorioallantoic membrane (CAM) of SPF chick embryos (McNulty *et al*., 1987). The FP3 isolate of ELV-3 was isolated from the meconium of dead-in-shell chick embryos at the Central Veterinary Laboratory, now called Veterinary Laboratories Agency (VLA), Weybridge, UK (Spackman *et al*., 1984). Virus received from VLA (passage history unknown to us) was propagated by yolk sac inoculation of SPF chick embryos with clarified embryo liver suspensions, collected 5 days post inoculation, being used in this investigation. The 11672 isolate of ELV-3 is one of several viruses, which were isolated in this laboratory from weak chicks that were submitted as part of an investigation into hatchability problems. These viruses are considered to be additional ELV-3 isolates on the basis that they share a

close antigenic relationship to the FP3 ELV-3 isolate (unpublished results). The 11672 virus was isolated and propagated by yolk sac inoculation of SPF chick embryos. Clarified homogenates of whole embryos harvested between 3 and 5 days after inoculation with 6th passage virus were used. ELV-4 (isolate 612) was isolated in South Africa during an investigation of respiratory distress in broiler chickens (McNeilly *et al*. 1994). The virus was initially isolated and propagated in SPF chick embryos using yolk sac inoculation. For this investigation, virus that had been passaged twice in CAMs of 10-day-old SPF embryos was used.

The G4260 isolate of the avian nephritis virus serotype 1 (ANV-1) was supplied by Dr S. Yamiguchi, Poultry Disease Laboratory, National Institute, Kurachi, Japan. The virus was passed 6 times in chick kidney cells and the virus pool used in this investigation was then prepared by inoculating CAMs of 10-day-old SPF chick embryos. The P22 18.8.00 isolate of CAstV, hereafter named the reference CAstV isolate, was obtained from Dr I. Tarpey, Intervet, Milton Keynes, UK. This virus, which was originally isolated and propagated in chick embryo liver (CEL) cells (Baxendale & Mebatsion, 2004), was passed twice in CEL cells before use in this investigation.

DHV-2 (isolate M52), which was kindly supplied by Mr. R.E. Gough, VLA, was passaged twice in CAMs of 10-day-old SPF embryos before use in this investigation. DHV-3 (isolate X1222A Calnek) was obtained from Dr. B.W. Calnek, Cornell University, New York, USA. This virus was passaged once through 1-day-old ducklings and the virus pool used in this investigation was derived by inoculating clarified liver suspension onto the CAMs of 10-day-old duck embryos.

Antisera. Antisera specific to ELV-3 (isolate FP3) and ELV-4 (612 isolate) were prepared by experimental infection of 6-week-old SPF chickens as described previously (McNulty *et al*., 1990; McNeilly *et al*. 1994). Antiserum to the 11672 ELV-3 isolate was prepared by collecting serum from 5-week-old SPF chickens that had been experimentally infected at 1-day-old with embryo-grown virus using the oral route of inoculation.

Immunofluorescence. Indirect immunofluorescence (IIF) was performed as described by McNulty *et al*. (1990). This involved inoculating chick embryo liver (CEL) cells, grown for 24 h on circular glass coverslips (diameter 13 mm), with virus pools including isolates FP3 and 11672 of ELV-3 and the reference CAstV isolate. Chick embryo kidney (CEK) cells grown on coverslips were infected with the 612 isolate (ELV-4). The infected coverslip cultures were harvested after 48 h incubation at 37°C, fixed in acetone and stored at -20°C until used. Fixed coverslip cultures were incubated for 1 h at 37°C with dilutions of antisera. After washing bound chicken antibody was reacted with fluoroscein isothyocyanate (FITC) - conjugated goat anti-chicken Ig (usually 1:100) for 1 h at 37°C. Immunofluorescent staining was detected using a UV microscope.

Reverse transcription polymerase chain reaction (RT-PCR). Viral RNA was extracted from 200 µl samples using the RNeasy Mini Kit (Qiagen, Crawley, West Sussex). Two RT-PCR methods, each employing pairs of degenerate oligonucleotide primers (Invitrogen, Paisley, UK), were used to attempt to amplify astrovirus-specific cDNA fragments from RNA samples of ELVs, ANV-1, DHV-2 and DHV-3 using the SuperScript III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase Kit (Invitrogen). Method 1 used forward primer 5′-GAYTGGACIMGITAYGAYGGIACIATICC-3′ and reverse primer 5′-YTTIACCCACATICCRAA-3′ and amplified a fragment of approximately 434 nt, which corresponded to nt 3799-4233 in the genome of the G4260 strain of ANV-1 (accession number AB033998). The RT-PCR conditions were initial denaturation, 94°C, 5 min, then 45 cycles of denaturation, 94°C, 1 min; annealing, 45°C, 1 min; extension, 72°C, 90 sec followed by a final extension step, 72°C, 5 min. Method 2 was that described by Tang *et al*. (2005a). Briefly, this resulted in the amplification of a 597 bp fragment, which corresponded to nt 3550-4147 of the ANV-1 sequence. The amplification products were examined by electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualised by UV transillumination.

Cloning, sequencing and sequence analysis. Fragments amplified by RT-PCR method 1 were cloned using the pCR2.1®TOPO vector (Invitrogen). The inserts from 2 clones were

sequenced for each amplicon using the M13 forward and reverse primers (ABI PRISM dye terminator ready reaction kit). Sequences were analysed using the Vector NTI software suite (Invitrogen) and searches were performed by the BLASTN program using the non-redundant nucleic acid sequence database at the NCBI, USA. Sequence alignments were performed using the LALIGN and Clustal W programs (University of Wisconsin Genetics Group version 8). A Neighbor-joining tree was constructed based on alignments of the partial (~130 aa) ORF1b translated amino acid sequences of avastroviruses and mamastroviruses (Figure 3). A distance matrix was constructed using the JTT matrix-based model of amino acid substitutions (Jones *et al*., 1992). A second Neighbor-joining tree was constructed based on alignments of the partial (319 nt) ORF1b sequences of avastroviruses, with a single mamastrovirus (HAstV-3) being included as an outgroup (Figure 4a, b). The 319 nt sequence comprised that part of the 434 nt amplicon sequence (determined this paper), which overlapped ORF 1b avastrovirus sequences submitted to the databases. In total database sequences from 127 TAstV-2, 7 TAstV-1, 13 ANV and 13 CAstV sequences were used in the comparison with the ELVs, reference CAstV and DHVs. A distance matrix was constructed using the Kimura 2-parameter model of nucleotide substitutions (Kimura, 1980). All analyses were performed using MEGA 4.0. (Tamura *et al*., 2007).

Genbank Accession numbers. The accession numbers for the astrovirus ORF 1b sequences (~390 nt) amplified by the Method 1 degenerate RT-PCR are as follows: EU668998 (11672 isolate of ELV-3), EU668999 (FP3 isolate of ELV-3), EU669000 (P22 18.8.00 isolate of CAstV), EU669001 (612 isolate of ELV-4), EU669002 (EF91-276 isolate of avian nephritis virus) EU669003 (M52 strain of duck hepatitis virus type 2) and EU669004 (X1222A Calnek isolate of duck hepatitis virus type 3). The accession numbers for other avian and mammalian astroviruses are shown within Figure 3 and Figure 4 a, b.

Results

Antigenic relationship of ELVs. The antigenic relationships of the FP3 and 11672 isolates of ELV-3, the 612 isolate of ELV-4 and the reference CAstV isolate were investigated by indirect immunofluorescence (Table 1), using virus-infected coverslip CEL cultures and antisera prepared against the FP3, 11672 and 612 isolates. Intracytoplasmic staining was detected with all 4 virus isolates following reaction with the experimental antisera and the FITC-anti-chick Ig conjugate (Figure 1a, b). With some infected cells, brightly-staining, granular inclusions were observed, whereas other infected cells on the same coverslip displayed more evenly dispersed staining patterns. Infected cells showing very brightly-staining, granular foci were more commonly seen in CEL cells infected with ELV-3 isolates than in CEK cells infected with ELV-4. Reaction of FP3-infected and 11672-infected coverslip cultures with dilutions of homologous and heterologous antisera showed similar positive staining endpoints, indicating that these ELV-3 isolates were closely related antigenically. In contrast, antisera to the ELV-3 isolates reacted at low dilutions only with the 612 isolate of ELV-4, and antiserum to the 612 isolate reacted similarly with the FP3 and 11672 isolates. Although antisera to the 11672, FP3 and 612 isolates produced virus-specific staining with the reference CAstV, the antiserum to the 612 isolate produced positive staining at a higher dilution with the reference CAstV (1:1024 dilution) than with the 11672 (1:64 dilution) and FP3 (<1:64 dilution) isolates, indicating that the reference CAstV isolate was more closely related antigenically to the 612 isolate of ELV-4 than to the ELV-3 isolates.

Detection of astrovirus-specific cDNAs in ELV samples. DNA fragments of approximately 434 bp were amplified when RNAs extracted from ANV-1, the reference CAstV, ELV-1, ELV-3, ELV-4, DHV-2 and DHV-3 were subjected to RT-PCR performed using Method 1 (data not shown). Application of Method 2 RT-PCR produced fragments of approximately 597 bp for the same samples except the two isolates of ELV-3. No PCR products were produced by either method with samples of ELV-2. Following cloning and sequencing of the 434 bp RT-PCR products, blast search analyses with the predicted amino acid sequences revealed that the ELV-1, ELV-3, ELV-4, DHV-2 and DHV-3 were most closely related to the

avian astroviruses ANV-1, turkey astrovirus type 1 (TAstV-1) and turkey astrovirus type (TAstV-2).

Comparative sequence analyses. Pairwise comparisons based on the nucleotide and predicted amino acid sequences (~130 amino acids) of part of the RNA polymerase gene encompassed by the Method 1 RT-PCR primers were undertaken to determine the relationships of the 3 ELVs and the DHVs to previously sequenced avian astroviruses (Table 2).

High amino acid identity values were shared by ELV-1 and ANV-1 (97%), by ELV-4 (isolate 612) and the reference CAstV (95%) and by the two ELV-3 isolates, FP3 and 11672 (98%), indicating that the viruses within each pair were closely related. The % identities shared by the ELV-3 isolates with the other 4 avian astroviruses were 48% (ANV-1/ ELV-1), 84-85% (reference CAstV/ ELV-4), 58-59% (TAstV-1), 70-71% (TAstV-2), 72-73% (DHV-2) and 72% (DHV-3). DHV-2 shared similar levels (72-73%) of amino acid identity with reference CAstV/ ELV-4, the ELV-3s and TAstV-2, and was less closely related to TAstV-1 (58%) and ANV-1/ELV-1 (53%), while DHV-3 was most closely related to TAstV-2 (81%). The levels of amino acid identity shared by ANV-1 and ELV-1 isolates with the other avian astroviruses were generally lower, ranging from 46% (TAstV-1) to 53% (TAstV-2).

In most cases the nucleotide identity values were lower than those obtained using amino acid comparisons. Thus, nucleotide identities of 93%, 87% and 95% were displayed by the ANV-1/ ELV-1 pair, the reference CAstV/ ELV-4 pair and the ELV-3 pair, respectively. The reference CAstV/ ELV-4 pair shared 76-79% nucleotide identity with the ELV-3 isolates. The ANV-1/ ELV-1 isolates were the least closely related to the other astroviruses, sharing nucleotide identities of between 53% and 57%.

Alignment of the predicted ~130 amino acid sequences contained by the 434 bp amplicons identified a number of conserved amino acid sequences (Figure 2). These included GNPSGQFSTT (residues 1324 to 1333 within the ANV-1 ORF 1b) and YGDDRL (residues 1374 to 1379 within the ANV-1 ORF 1b), which contains the YGDD motif that is associated with viral RNA dependent RNA polymerases. Alignment of the nucleotide sequences

revealed very little conserved sequences, the longest conserved sequence being six nucleotides (data not shown).

Phylogenetic analyses. A Neighbor-joining tree was constructed based on ~130 amino acid sequences, in which the ELVs, DHVs and the reference CAstV isolate were compared with other avian and mammalian astroviruses for which corresponding sequences were available (Figure 3). Examination showed that the 10 mammalian and 20 avian astroviruses formed two major groupings. Within the mamastrovirus grouping, isolates representing 6 human astrovirus serotypes were most closely related. Within the avastrovirus grouping, the 11 TAstV-2 isolates were very closely related to one another and most distantly related to ANV and ELV-1, folowed by TAstV-1. Within the CAstV grouping, the closely related ELV-3 CAstVs were separate from ELV-4 and the reference CAstV isolates. Whereas DHV-3 was more closely related to the TAstV-2 isolates, DHV-2 was more closely related to the CAstVs.

A second Neighbor-joining tree based on alignments of 319 nt sequences derived from 167 avian astroviruses showed separate clades for TAstV-2 (127 sequences), TAstV-1 (7 sequences), ANV (14 sequences) and CAstV (17 sequences) (Figure 4a, b; The complete phylogenetic tree showing all 167 avian astrovirus sequences is available as supplementary data by accessing……). Close examination indicated that ELV-1 was grouped within the ANV clade, where it was closely related to the ANV-1 isolate. The CAstV clade comprised two major groupings. Thus, the ELV-3 CAstV isolates were grouped together and were more distantly related to the other CAstVs, which included ELV-4 and the reference CAstV isolate (P22 18.800). The DHV-2 and DHV-3 isolates were separately grouped, with DHV-3 being more closely related to the TAstV-2 clade and DHV-2 being more closely related to the CAstVs than to the other avastroviruses. The TAstV-2 clade, shown collapsed in Figure 4a and fully presented in Figure 4b, comprised a number of major sub-groupings.

Discussion

This paper reports the identification of three previously described, antigenically different chicken ELV isolates as viruses that can be classified within the genus *Avastrovirus* of the

family *Astroviridae*. On the basis of antigenic and genetic relatedness shared with a reference CAstV isolate, first described by Baxendale & Mebatsion (2004), the ELV-3 and ELV-4 isolates can be regarded as different isolates of CAstV. Based on ORF 1b sequence comparisons this work has also shown that ELV-1 represents an isolate of ANV, that the astrovirus DHV-2 is distinct from other avastroviruses, and that DHV-3 can also be identified an avian astrovirus.

The method used for identification involved application of a degenerate primer-based astrovirus-specific RT-PCR to amplify cDNA fragments from ELV samples in combination with nucleotide sequencing and analysis. The design of the degenerate primers was based on the occurrence of semi-conserved nucleotide sequences found within the RNA dependent RNA polymerase gene (ORF 1b) genes of ANV-1, TAstV-1 and TAstV-2. The RT-PCR method (Method 1), which produced a cDNA product of approximately 434 bp, corresponding to nt 3799-4233 in the ANV-1 sequence, succeeded in amplifying astrovirus cDNA fragments from the majority of ELV isolates tested as well as from samples of DHV-2 and DHV-3. In this investigation we found that an alternative degenerate primer-based astrovirus-specific RT-PCR approach (Method 2), described originally by Tang *et al*. (2005a), which amplified a cDNA product of approximately 597 bp corresponding to nt 3550-4147 in the ANV-1 sequence, failed to produce a PCR product from the two ELV-3 isolates tested. Failure of both RT-PCR methods to produce astrovirus-specific PCR products with samples of ELV-2 suggests that this ELV may belong to another virus family or may be an astrovirus exhibiting greater sequence diversity.

Two different astrovirus species have been identified in chickens. Avian nephritis virus (ANV), originally regarded as a picornavirus, was characterised as the first astrovirus of chickens on the basis of nucleotide sequence and genome organisational similarities shared with other astroviruses (Imada *et al*., 2000). ANV is associated with nephritis in young chickens but infections have also been shown to cause growth depression (Shirai *et al*., 1991a, b). Evidence supports the existence of at least 2 serotypes of ANV and different pathotypes (Shirai *et al*., 1991b). An earlier study using indirect immunofluorescence and serum neutralization tests showed that ELV-1 was closely related antigenically to the G4260 serotype 1 strain of ANV (ANV-1; McNulty *et al*., 1990). In the present paper, sequence

comparisons of the small ORF 1b region confirmed that ELV-1 shared high nucleotide (92%) and amino acid (97%) identities with ANV-1 (Table 2), and phylogenetic analyses showed that ELV-1 was grouped with other ANVs (Figure 3, 4a). However, sequence comparisons involving other genome regions including the capsid protein gene will be required to determine the exact relationship of ELV-1 to ANV-1 and other ANV isolates. Based on sequence comparisons of part of the non-structural protease gene (ORF 1a), Mandoliki *et al*. (2006) have recently shown that ANV can be grouped into three genotypes.

CAstV was identified as the second astrovirus of chickens by Baxendale & Mebatsion (2004), who described the isolation and characterisation of 3 antigenically similar viruses from broiler flocks with runting problems. CAstVs were also detected in a recent study by Pantin-Jackwood *et al*. (2006), who applied the Method 2 RT-PCR to faeces and gut content samples from chickens and turkeys obtained from problem and non-problem USA flocks. Nucleotide identities between 85.1% and 98.4% and amino acid identities between 92.4% and 100% were observed when 13 CAstV ORF 1b amplicon sequences were compared. The phylogenetic analysis, based on the comparison of common 319 nucleotide ORF 1b sequences, demonstrated that, whereas the reference CAstV and ELV-4 isolates were closely grouped with the thirteen USA CAstVs, the two ELV-3 isolates formed a separate, less closely related CAstV grouping (Figure 4a). This finding was consistent with the phylogenetic tree based on amino acid sequences (Figure 3) and the pairwise comparison results presented in Table 2, which showed that the ELV-3 isolates shared nucleotide and amino acid identities of 76-79% and 84-85% respectively with the ELV-4/ reference CAstV isolate pair. On the basis that greater diversities were observed when other avian astrovirus species are compared over the same ORF 1b region, these identity levels do not warrant identifying ELV-3 as a separate CAstV species. A recent paper describing the genomic analyses of nine TAstV-2-like clinical isolates demonstrated the importance of comparing different genomic regions in phylogenetic analyses and reported data, which indicated that recombination between sequence-distinct isolates can occur at different locations throughout the genome (Strain *et al.*, 2008). Therefore, sequence data from other genome regions including ORF 1b (non-structural protease), and ORF 2 (capsid protein) are required before the taxonomic relationship of the ELV-3 CAstVs to other CAstV isolates is established.

Data relating to the immunogenicity of avian astroviruses is lacking. Although the antigenic relatedness detected by IIF suggested that the ELV-3s, ELV-4 and the reference CAstV belonged to the same astrovirus species (Table 1), the differences in the levels of IIF cross-reactivity are difficult to interpret given that polyclonal antisera produced in experimentally infected chickens were used. For example, it is possible that antibodies specific to common epitopes on both the non-structural and structural proteins are contributing to the antigenic cross-reactivity. Work with human astroviruses has demonstrated the existence of a common and serotype-specific epitopes within capsid proteins when different serotypes were compared (van Hemert *et al.,* 2007) and this is likely to be the case with avian astroviruses. The levels of IIF cross-reactivity observed between the ELV-3 CAstVs and the ELV-4 CAstV were similar to those detected when ANV isolates belonging to 2 different serotypes were compared (Shirai *et al*., 1991b), suggesting that the two ELV-3 CAstV isolates (11672 and FP3) may be serotypically different from the ELV-4 and reference CAstVs. Serotyping ELV and CAstV isolates using cross-neutralization tests has not been possible due to the very poor growth of these virus isolates in cell culture. For this reason, the levels of CAstV antigenic variation may have to be deduced from capsid protein sequences.

Although DHV-2 has been classified as an astrovirus for many years on the basis of virion morphology (Gough *et al*., 1985), this is the first paper to report nucleotide sequence specific to DHV-2. We have presented evidence that DHV-3 can also be identified as a second duck astrovirus. Although it is acknowledged that the sequences of other genomic regions should be considered before phylogenetic and taxonomic relationships are established, the levels of nucleotide (64%) and amino acid (69%) identities shared by DHV-2 and DHV-3 in the ORF 1b sequences compared supported the view that these isolates may represent different species. Interestingly, our analyses based on the ORF 1b region indicated that DHV-3 was more closely related to TAstV-2 than DHV-2, which appeared to be more closely related to the CAstVs (Figure 3, 4).

The sequence data presented in this paper has allowed ELVs and DHV-3 to be identified as astroviruses, and has allowed a preliminary phylogenetic study based on ORF 1b sequences of all known avian astroviruses to be undertaken. The sequences now available provide starting points from which larger regions of the chicken and duck astrovirus genomes

can be cloned and sequenced. The availability of additional virus-specific sequences will facilitate the development of molecular diagnostic tests, with which the roles of astroviruses as disease-causing agents can be investigated.

Acknowledgements. This work was funded in part by the BBSRC, DEFRA and the Department of Agriculture and Rural Development for Northern Ireland.

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Table 1. *Antigenic relatedness of ELV isolates as determined by cross-indirect immunofluorescence testing.*

Virus	Antiserum		
	ELV-3 (11672)	ELV-3 (FP3)	ELV-4 (612)
ELV-3 (11672)	1024[b]	128	64
ELV-3 (FP3)	1024	256	<64[c]
ELV-4 (612)	32	16	>4096
Reference CAstV[a]	256	32	1024

a: Antiserum was not available to the reference CAstV isolate

b: Reciprocal of the highest dilution showing virus-specific staining

c: Non-specific staining prevented the detection of virus-specific staining at lower dilutions.

Table 2: *Pairwise amino acid (top right) and nucleotide (bottom left) identity comparisons of chicken, turkey and duck astroviruses and ELVs*

	ANV-1	ELV-1	ELV-4 612	CAstV ref	ELV-3 FP3	ELV-3 11672	TAstV-1	TAstV-2	DHV-2	DHV-3
ANV-1	-	97	50	50	48	48	46	52	53	47
ELV-1	93	-	50	50	48	48	46	52	53	47
ELV-4 612	57	56	-	95	85	85	58	70	72	69
CAstV ref	55	54	87	-	84	85	57	70	73	68
ELV-3 FP3.	53	52	77	77	-	98	59	71	72	72
ELV-3 11672	53	52	76	79	95	-	58	70	73	72
TAstV-1	55	55	60	58	61	63	-	58	58	55
TAstV-2	56	56	61	60	62	61	59	-	73	81
DHV-2	55	56	66	68	66	66	58	67	-	69
DHV-3	55	52	64	63	63	64	59	73	64	-

Legends

Figure 1. *Detection of ELV antigen by indirect immunofluorescence. (a) Chick embryo liver cells infected with ELV-3; (b) Chick embryo kidney cells infected with ELV-4. Fluorescent staining is observed in the cytoplasm of virus-infected cells, sometimes as brightly stained granular foci and sometimes with a more evenly dispersed appearance.*

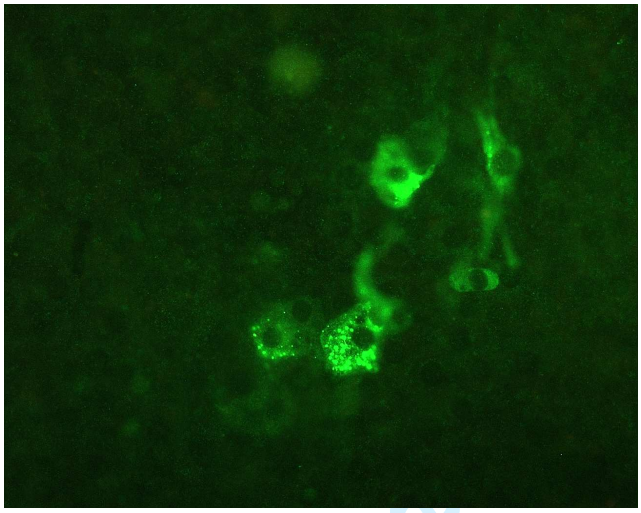
Figure 2. *Multiple alignment of the predicted amino acid sequence within ORF 1b in selected avian astroviruses and reference ANV-1. Asterisks denote amino acids in that column are identical in all sequences in the alignment. Colons indicate conserved substitutions and full stops represent semi-conserved substitutions. The multiple alignment was generated using the ClustalW2 program at EMBL-EBI (available at www.ebi.ac.uk/clustalw/). The terms ELV-3a and ELV-3b relate to the FP3 and 11672 isolates respectively.*

Figure 3. *Neighbor-joining tree based on alignments of the partial (~130 amino acid) ORF1b translated amino acid sequences of avastroviruses and mamastroviruses. A distance matrix was constructed using the JTT matrix-based model of amino acid substitutions (Jones et al., 1992). The results of 1000 bootstrap pseudo-replicates are shown as percentage values on the branches (only values of 70% and above are shown). All analyses were performed with MEGA 4.0. The mamastroviruses included human astrovirus (HAstV) serotypes 1, 2, 3, 4, 5 and 8, cheetal astrovirus (CheetahAstV), bat astrovirus (BatAstV), mink astrovirus (MAstV) and ovine astrovirus (OAstV).*

Figure 4. *Neighbor-joining tree based on alignments of the partial (319 nt) ORF1b sequences of avastroviruses. A single mamastrovirus (HAstV-3) was included as an outgroup. A distance matrix was constructed using the Kimura 2-parameter model of nucleotide substitutions (Kimura, 1980). The results of 1000 bootstrap pseudo-replicates are shown as percentage values on the branches (only values of 70% and above are shown). All analyses were performed with MEGA 4.0. In Figure 4a, the clade comprising 127 TAstV-2 sequences was collapsed to allow the groupings of the other avastroviruses to be more clearly examined.*

Figure 4b shows the TAstV-2 clade. (The complete phylogenetic tree showing all 167 avian astrovirus sequences is available as supplementary data by accessing……).

Fig 1 a:



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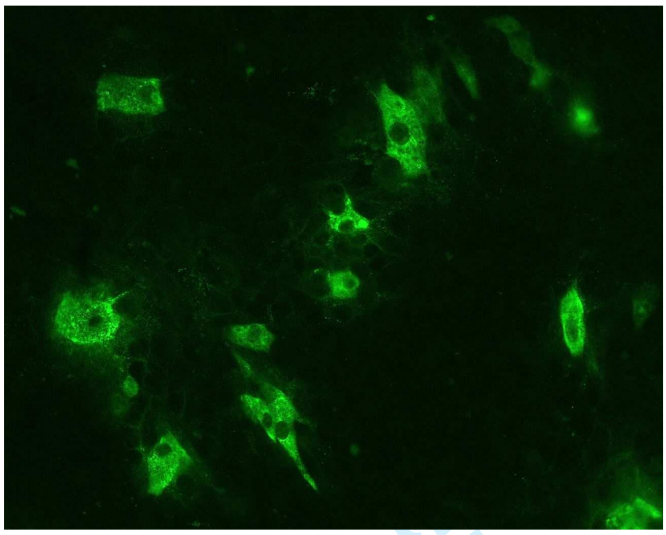

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DHV-2           TTVDNNMVNCWLTNFELAYLHNKQKGRLPTVKELRENSAFICYGDDRLLSVSEDFVK-YE 119
TAstV-2         TTVDNNMINVWLTTFEVSYLFFKQRGRLPTEKELQENCSMICYGDDRLLSIRKGFVE-YE 119
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ELV-3a          PDTVVAMYADV 130
ELV-3b          PETVVAMYADV 130
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TAstV-2         PDTVIDMYKNI 130
DHV-3           IDTVVDMYREV 129
TAstV-1         TAAVIAMYKDV 131
ANV-1           PQVTIDMYKNI 129
ELV-1           SQVTIDMYKNI 129
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Figure 2. *Multiple alignment of the predicted amino acid sequence within ORF 1b in selected avian astroviruses and reference ANV-1. Asterisks denote amino acids in that column are identical in all sequences in the alignment. Colons indicate conserved substitutions and full stops represent semi-conserved substitutions. The multiple alignment was generated using the ClustalW2 program at EMBL-EBI (available at www.ebi.ac.uk/clustalw/). The terms ELV-3a and ELV-3b relate to the FP3 and 11672 isolates respectively.*

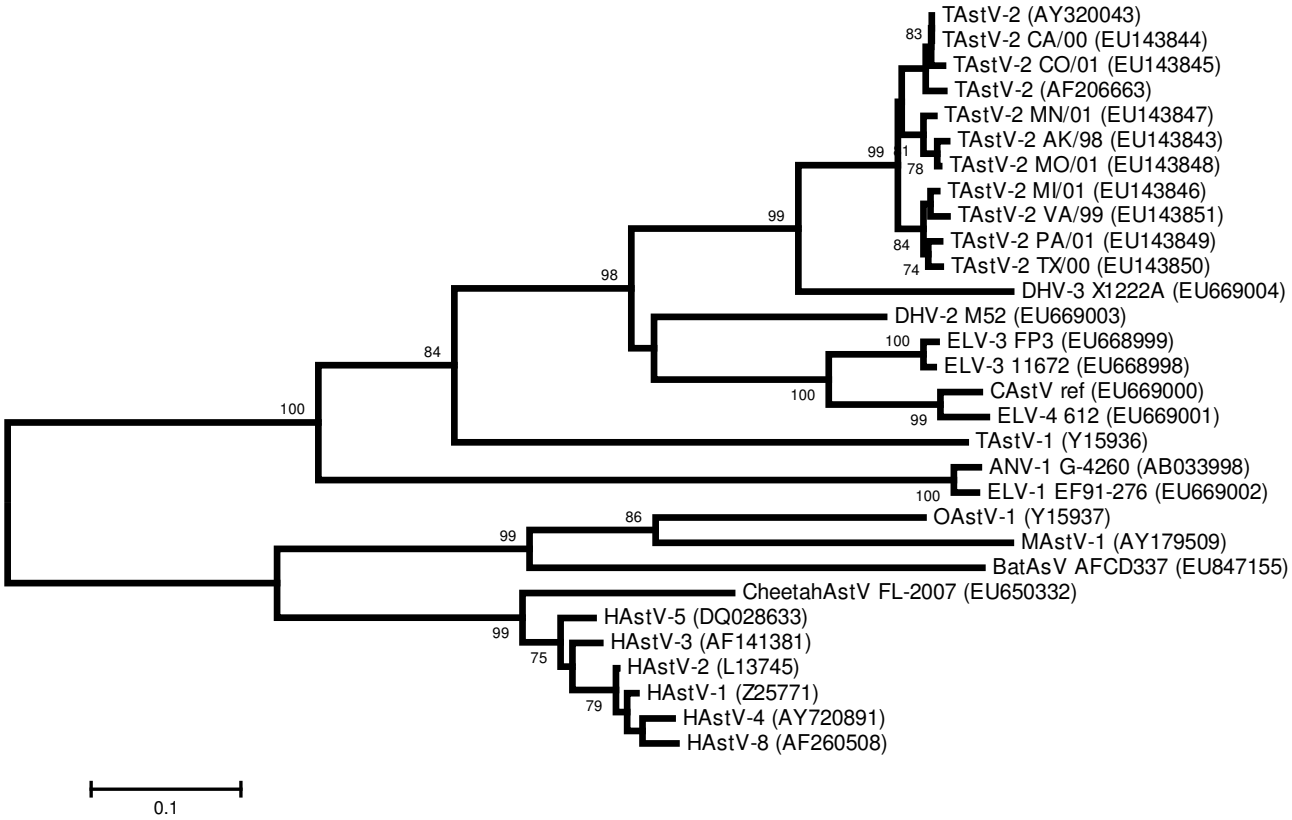
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Fig 3.

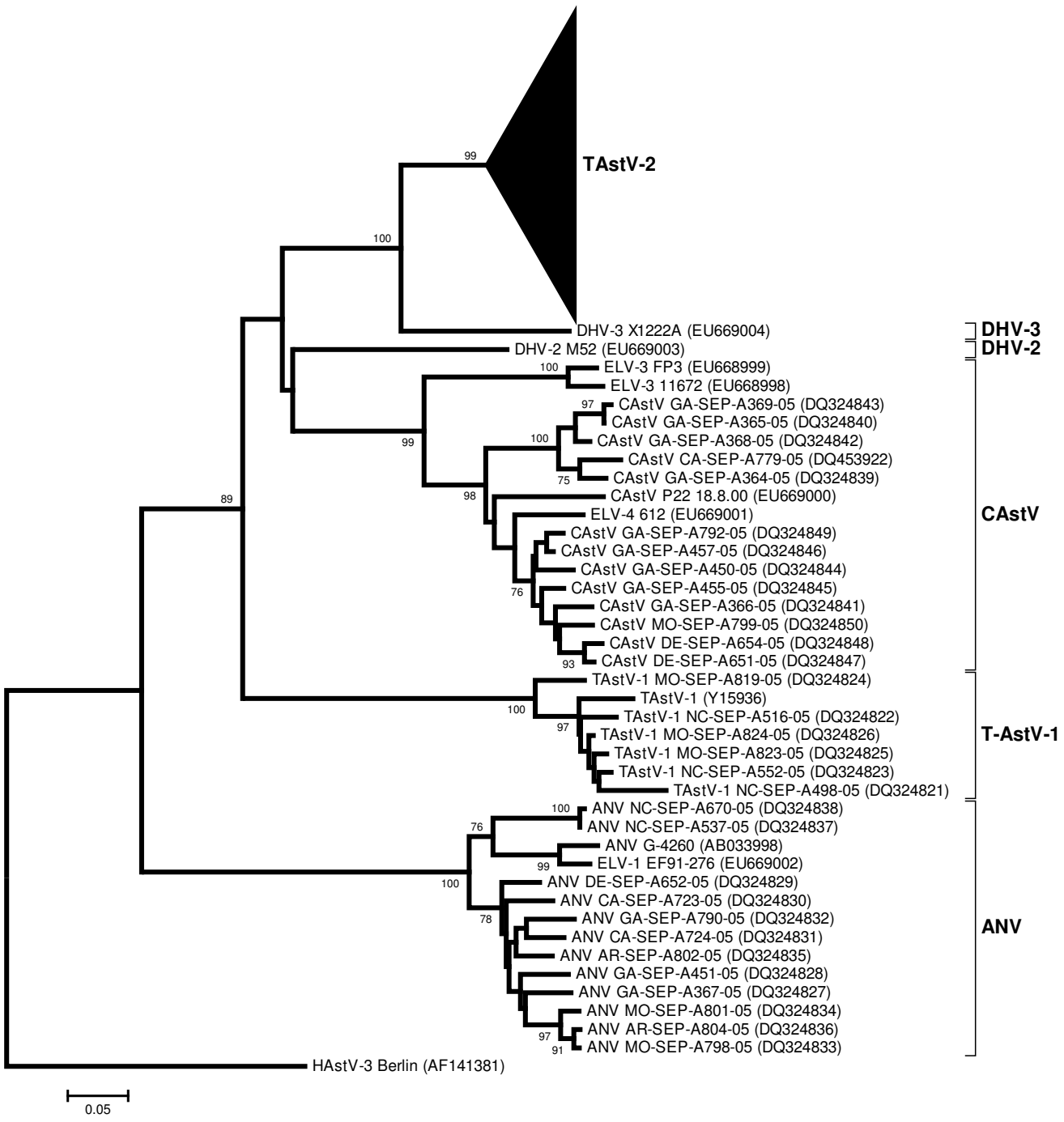
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Fig 4 a

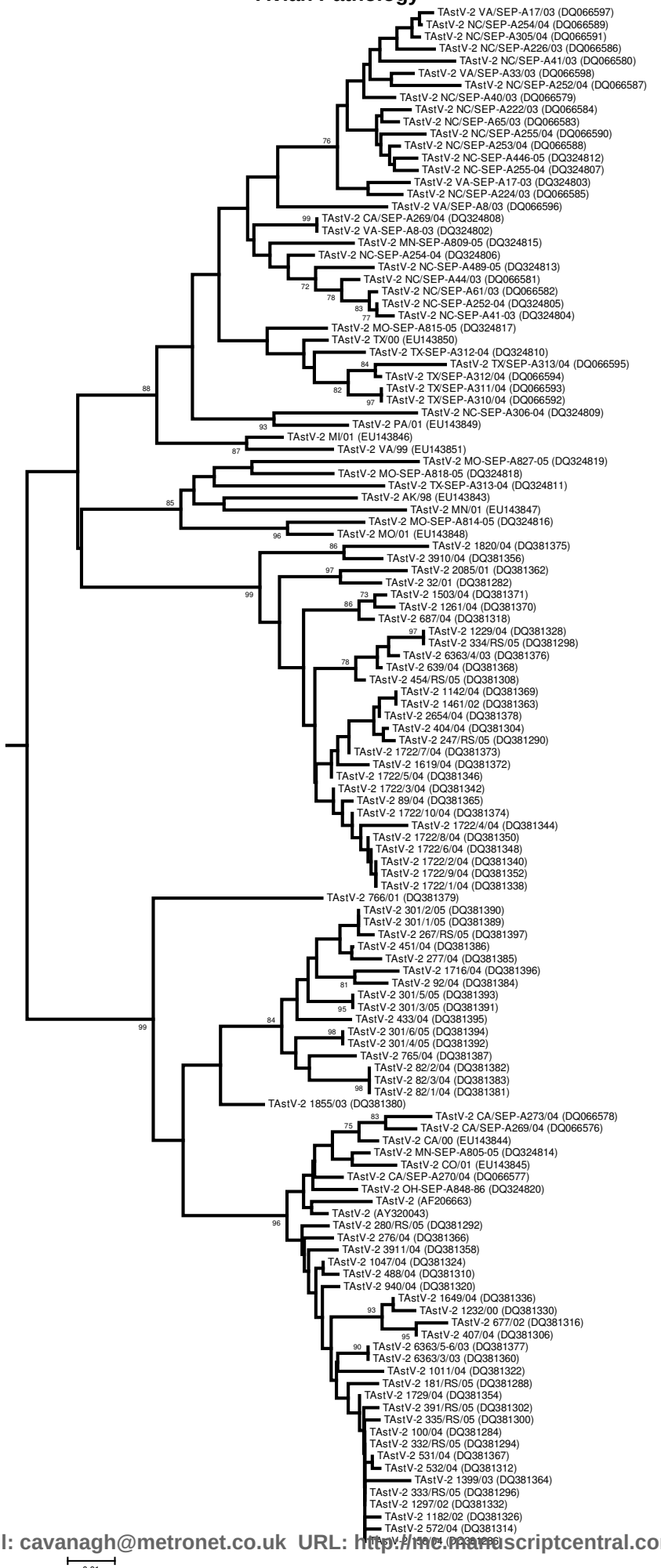
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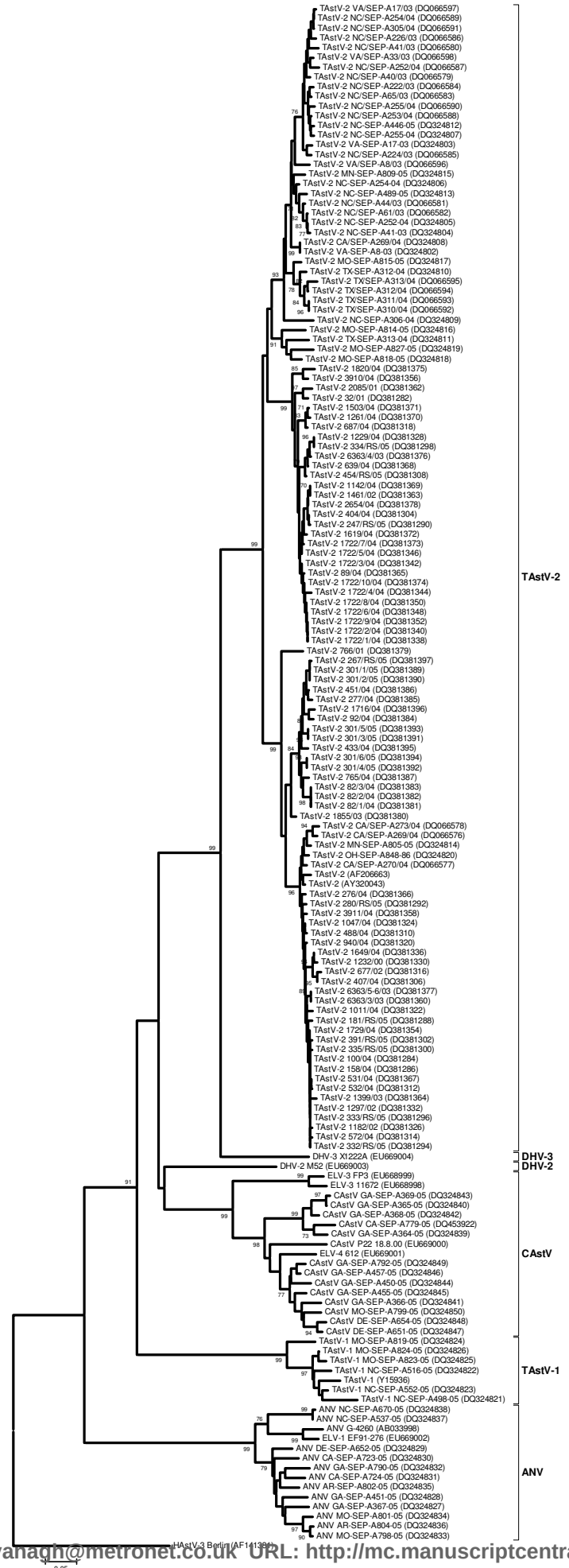
Fig 4 b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TAstV-2 NC-SEP-A489-05 (DQ324813)
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TAstV-2 CA/SEP-A269/04 (DQ324808)
TAstV-2 VA-SEP-A8-03 (DQ324802)
TAstV-2 MO-SEP-A815-05 (DQ324817)
TAstV-2 TX-SEP-A312-04 (DQ324810)
TAstV-2 TX/SEP-A313/04 (DQ066595)
TAstV-2 TX/SEP-A312/04 (DQ066594)
TAstV-2 TX/SEP-A311/04 (DQ066593)
TAstV-2 TX/SEP-A310/04 (DQ066592)
TAstV-2 NC-SEP-A306-04 (DQ324809)
TAstV-2 MO-SEP-A814-05 (DQ324816)
TAstV-2 TX-SEP-A313-04 (DQ324811)
TAstV-2 MO-SEP-A827-05 (DQ324819)
TAstV-2 MO-SEP-A818-05 (DQ324818)
TAstV-2 1820/04 (DQ381375)
TAstV-2 3910/04 (DQ381356)
TAstV-2 2085/01 (DQ381362)
TAstV-2 32/01 (DQ381282)
TAstV-2 1503/04 (DQ381371)
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TAstV-2 1229/04 (DQ381328)
TAstV-2 334/RS/05 (DQ381298)
TAstV-2 6363/4/03 (DQ381376)
TAstV-2 639/04 (DQ381368)
TAstV-2 454/RS/05 (DQ381308)
TAstV-2 1142/04 (DQ381369)
TAstV-2 1461/02 (DQ381363)
TAstV-2 2654/04 (DQ381378)
TAstV-2 404/04 (DQ381304)
TAstV-2 247/RS/05 (DQ381290)
TAstV-2 1619/04 (DQ381372)
TAstV-2 1722/7/04 (DQ381373)
TAstV-2 1722/5/04 (DQ381346)
TAstV-2 1722/3/04 (DQ381342)
TAstV-2 89/04 (DQ381365)
TAstV-2 1722/10/04 (DQ381374)
TAstV-2 1722/4/04 (DQ381344)
TAstV-2 1722/8/04 (DQ381350)
TAstV-2 1722/6/04 (DQ381348)
TAstV-2 1722/9/04 (DQ381352)
TAstV-2 1722/2/04 (DQ381340)
TAstV-2 1722/1/04 (DQ381338)
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TAstV-2 766/01 (DQ381379)
TAstV-2 267/RS/05 (DQ381397)
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TAstV-2 301/2/05 (DQ381390)
TAstV-2 451/04 (DQ381386)
TAstV-2 277/04 (DQ381385)
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TAstV-2 433/04 (DQ381395)
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TAstV-2 301/4/05 (DQ381392)
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TAstV-2 (AY320043)
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TAstV-2 280/RS/05 (DQ381292)
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TAstV-2 391/RS/05 (DQ381302)
TAstV-2 335/RS/05 (DQ381300)
TAstV-2 100/04 (DQ381284)
TAstV-2 158/04 (DQ381286)
TAstV-2 531/04 (DQ381367)
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TAstV-2 1399/03 (DQ381364)
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TAstV-2 333/RS/05 (DQ381296)
TAstV-2 1182/02 (DQ381326)
TAstV-2 572/04 (DQ381314)
TAstV-2 332/RS/05 (DQ381294)
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DHV-3
DHV-2 M52 (EU669003)
DHV-2
ELV-3 FP3 (EU668999)
ELV-3 11672 (EU668998)
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CAstV GA-SEP-A365-05 (DQ324840)
CAstV GA-SEP-A368-05 (DQ324842)
CAstV CA-SEP-A779-05 (DQ453922)
CAstV GA-SEP-A364-05 (DQ324839)
CAstV P22 18.8.00 (EU669000)
ELV-4 612 (EU669001)
CAstV GA-SEP-A792-05 (DQ324849)
CAstV GA-SEP-A457-05 (DQ324846)
CAstV GA-SEP-A450-05 (DQ324844)
CAstV GA-SEP-A455-05 (DQ324845)
CAstV GA-SEP-A366-05 (DQ324841)
CAstV MO-SEP-A799-05 (DQ324850)
CAstV DE-SEP-A654-05 (DQ324848)
CAstV DE-SEP-A651-05 (DQ324847)
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TAstV-1 (Y15936)
TAstV-1 NC-SEP-A552-05 (DQ324823)
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TAstV-1
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ANV NC-SEP-A537-05 (DQ324837)
ANV G-4260 (AB033998)
ELV-1 EF91-276 (EU669002)
ANV DE-SEP-A652-05 (DQ324829)
ANV CA-SEP-A723-05 (DQ324830)
ANV GA-SEP-A790-05 (DQ324832)
ANV CA-SEP-A724-05 (DQ324831)
ANV AR-SEP-A802-05 (DQ324835)
ANV GA-SEP-A451-05 (DQ324828)
ANV GA-SEP-A367-05 (DQ324827)
ANV MO-SEP-A801-05 (DQ324834)
ANV AR-SEP-A804-05 (DQ324836)
ANV MO-SEP-A798-05 (DQ324833)
ANV
HAstV-3 Berlin (AF141381)
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